IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
REQUEST FOR FILING NATIONAL PATENT APPLICATION
Under 35 USC 111(a) and Rule 53(b)

PATENT APPLICATION

Hon. Commissioner of Patents
Washington, D.C. 20231

WITH SIGNED DECLARATION

NONPROVISIONAL
NON REISSUE
NON PCT NAT PHASE

12/18/00 U.S. PTO

JC784 U.S. PTO 09/737476 12/18/00

Herewith is the PATENT APPLICATION of
Inventor(s): FRENKEN, Leo et al

Title PRODUCTION OF ANTIBODIES

Atty. Dkt.: PM 275850 (M#) | T7060C (Client Ref)

including: Date: MONDAY, December 18, 2000

1. Specification: 62 pages (only spec. and claims) 2. ☐ Specification in non-English language
3. Declaration ☒ Original ☐ Facsimile/Copy ☒ Abstract 1 page(s); 13 numbered claims
4. ☐ Drawings: _____ sheet(s) ☐ informal; ☐ formal of size: ☐ A4 ☐ 11"
5. ☐ See top first page re prior Provisional, National or International application(s). ("X" box only if info is there and do not complete corresponding item 5 or 6). (Prior M# _____ SN _____)
6. **AMEND the specification** please by inserting before the first line: -- This is a ☐ Continuation-in-Part ☐ Divisional ☐ Continuation ☐ Substitute Application (MPEP 201.09) of:
6(a) ☐ National Appln. No. _____/_____ filed _____. (M# _____)
6(b) ☐ International Appln. No. _____ filed _____
which designated the U.S., and that International Application ☐ was ☐ was not published under PCT Article 21(2) in English.--
7. ☐ **AMEND the specification** by inserting before the first line: -- This application claims the benefit of U.S. Provisional Application No. 60/_____, filed _____. --
8. ☐ Attached is an assignment and cover sheet. Please return the recorded assignment to the undersigned.
9. ☐ Prior application is assigned to _____
by Assignment recorded _____ Reel _____ Frame _____
10. **FOREIGN** priority is claimed under 35 USC 119(a)-(d)/365(b) based on filing in EUROPE (country)
11.

Application No.	Filing Date	Application No.	Filing Date
(1) 99310188.0	17 DEC 1999	(2)	
(3)		(4)	
(5)		(6)	
(7)		(8)	
(9)		☐ See 3rd page for additional priorties	

12. 1 (No.) Certified copy (copies): ☒ attached; ☐ previously filed (date) _____
in U.S. Application No. _____/_____ filed on _____
13. Small entity status ⌀ ☒ is **not** claimed; ☐ is claimed (**Pre-filing confirmation required**)
13(a). ☐ Attached: _____ (No.) Small Entity Statement(s) (since 9/8/00 small entity statement(s) not essential to make claim)
13(b) ☐ See **NONPUBLICATION REQUEST** under Rule 213(a) attached (PAT-258)

14. **DOMESTIC/INTERNATIONAL** priority is claimed under 35 USC 119(e)/120/365(c) based on the following provisional, nonprovisional and/or PCT international application(s):

Application No.	Filing Date	Application No.	Filing Date
(1)		(4)	
(2)		(5)	
(3)		(6)	

15. ☐ This application is being filed under Rule 53(b)(2) since an inventor is named in the enclosed Declaration who was not named in the prior application.

16. ☒ Attached: SEQUENCE LISTING = Computer Readable and Paper Version (33 Pages)

17. ☐ Preliminary Amendment:

THE FOLLOWING FILING FEE IS BASED ON CLAIMS AS FILED LESS ANY ABOVE CANCELLED

				Large/Small Entity		Fee Code
18. Basic Filing Fee				$710/$355	$710	101/201
19. Total Effective Claims	16	minus 20 =	*0	x $18/$9 =	+ 0	103/203
20. Independent Claims	2	minus 3 =	*0	x $80/$40 =	+ 0	102/202
				*If answer is zero or less, enter "0"		
21. If any proper multiple dependent claim (ignore improper) is present , add (Leave this line blank if this is a reissue application)				+ $270/$135	+ 270	104/204
22.				TOTAL FILING FEE ENCLOSED =	$980	
23. If "non-English" box 2 is X'd, add Rule 17(k) processing fee				+ $130	+ 0	139
24. If "assignment" box 8 is X'd, add recording fee				+ $40	+ 0	581
25. ☐ Attached is a Petition/Fee under Rule No.				+ $130	+ 0	122
26.				TOTAL FEE ENCLOSED =	$980	

Our Deposit Account No. 03-3975

Our Order No. 60113 (C#) | 275850 (M#)

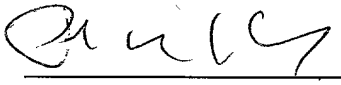
CHARGE STATEMENT: The Commissioner is hereby authorized to charge any fee specifically authorized hereafter, or any missing or insufficient fee(s) filed, or asserted to be filed, or which should have been filed herewith or concerning any paper filed hereafter, and which may be required under Rules 16-18 (missing or insufficient fee only) now or hereafter relative to this application and the resulting Official document under Rule 20, or credit any overpayment, to our Account/Order Nos. shown above for which purpose a duplicate copy of this sheet is attached.

This CHARGE STATEMENT does not authorize charge of the issue fee until/unless an issue fee transmittal form is filed.

Pillsbury Madison & Sutro LLP
Intellectual Property Group

1100 New York Avenue, NW
Ninth Floor
Washington, DC 20005-3918
Tel: (202) 861-3000
PNK/sdm

By Atty: Paul N. Kokulis **Reg. No.** 16773

Sig: 

Fax: (202) 822-0944
Tel: (202) 861-3503

NOTE: **File in duplicate with 2 post card receipts (PAT-103) & attachments**

APPLICATION UNDER UNITED STATES PATENT LAWS

Atty. Dkt. No. PM 275850
(M#)

Invention: PRODUCTION OF ANTIBODIES

Inventor (s): FRENKEN, Leo G.J.
VAN DER LOGT, Cornelis P.E.
TEH, Yin-Miin
VERHOEYEN, Martine E.
WILKINSON, Joy E.
JOBLING, Stephen A.

Pillsbury Madison & Sutro LLP
Intellectual Property Group
1100 New York Avenue, NW
Ninth Floor
Washington, DC 20005-3918
Attorneys
Telephone: (202) 861-3000

This is a:

- ☐ Provisional Application
- ☒ Regular Utility Application
- ☐ Continuing Application
  - ☒ The contents of the parent are incorporated by reference
- ☐ PCT National Phase Application
- ☐ Design Application
- ☐ Reissue Application
- ☐ Plant Application
- ☐ Substitute Specification
  Sub. Spec Filed ____________
  in App. No. ______/______
- ☐ Marked up Specification re
  Sub. Spec. filed ____________
  In App. No ______/______

SPECIFICATION

PRODUCTION OF ANTIBODIES

FIELD OF THE INVENTION

The present invention is in the field of applied biotechnology and relates in particular to an economic way of producing antibodies, or more particularly fragments thereof, in plants.

BACKGROUND OF THE INVENTION

The production of antibodies in microbial or plant systems can be advantageous for a number of applications.

Firstly microbial or plant sources can be used as bioreactors. In this situation the antibodies and/or their derivatives are produced on a large scale in modified organisms. The use of such 'bioreactors', especially plants has the advantage that scale up to produce large quantities e.g. more than 100 kg or even more than 1000 kg of antibodies is relatively easy and does not require significant investments in harvesting or processing equipment.

Alternatively antibodies and/or their derivatives can be produced in plants with the aim of reprogramming the plant metabolism or to improve defence mechanisms of said plant. The antibodies to be produced are chosen such that they target specific enzymes to modulate their activity and/or to provide the plant with protection against pathogens such as parasites and viruses.

Several attempts have been made to express antibodies or fragments thereof in micro-organisms or plants.

Evidence from the literature suggests that where it is desired to express antibodies in microbial systems, the most

favourable results are obtained using smaller fragments. Whole antibodies and larger antibody fragments, such as Fab fragments, are very difficult if not impossible to produce. Smaller molecules such as single chain antibody fragments (scFv) are somewhat easier. Generally, however the best results are obtained if even smaller molecules such as heavy chain variable fragments (HCV) are produced (see, for example, WO 94/25591 (Unilever)).

In plant host systems, both large molecules, such as complete murine antibodies and smaller murine antibody fragments such as single chain Fv (scFv) fragments are capable of being expressed.

The production of functional complete murine antibodies in plants was first reported by Hiatt et al, Nature, 342, 76-78 (1989). Subsequent reports of the functional expression of murine monoclonal antibodies in plants include Düring et al, Plant Molecular Biology, 15, 281-293, (1990) and Ma et al, European Journal of Immunology, 24, 131-138, (1994).

Transgenic tobacco plants expressing a synthetic gene encoding an antigen binding single chain Fv protein (scFv) and which produce functional scFv protein have been described by Owen et al, Biotechnology, 10, 790-794, (1992). The expression of functional (that is having antigen-binding activity) scFv protein in transgenic plants has also been described in other reports in the literature, see, for example, Tavlaoraki et al, Nature, 366, 469-472 (1993).

Given that the production of functional complete antibodies requires the correct assembly, via covalent and non-covalent interactions, of both the antibody heavy and light chains, it might have been expected that expression of smaller antibody fragments, with their less stringent assembly requirements,

would be advantageous. It has however been reported that generally, in practice, better yields are achieved with plants transformed with complete murine antibodies rather than small fragments (Ma et al, Science, 268, 716-719 (1995))

Furthermore, it is the experience of the present inventors that the expression of genes encoding scFv proteins in plants is not reliably reproducible and hence such a process would not readily lend itself to large scale production. Moreover, expression of genes encoding scFv molecules in plants can have an undesirable effect on plant cell morphology.

Benevenuto et al, Plant Molecular Biology, 17, 865-874 (1991) describe attempts to express smaller isolated murine heavy chain variable domain antibody fragments in plants. Successful expression is reported but there is no indication that binding affinity is retained.

For many purposes, it is important to be able to target antibodies or fragments thereof to specific cellular compartments. For example, where maximisation of yield is desired, targeting to the ER or the apoplastic space can help by providing a less reducing, more stable environment than is present in the cytoplasm. On the other hand, where it is desired to interfere with the activity of specific enzymes, in order to engineer metabolic changes in a plant, it is necessary to ensure that the antibodies are directed to the particular compartment in which the enzyme is active.

In order to direct the expression of the desired protein to a specific cellular compartment within a plant cell, the antibody proteins are fused to specific targeting sequences (peptides) where required. Expression in the chloroplast requires an N-terminal chloroplast targeting sequence (generally termed transit peptide) which is cleaved off upon entry into the chloroplast

(reviewed in Keegstra & Cline (2000) Plant Cell 11, 557-570). There is little sequence homology among these transit peptides except that they are often rich in hydroxylated amino acids (eg serine), are deficient in acidic amino acids and they can vary in length from 30 to >100 amino acids. Various transit peptides fused to heterologous proteins have been used successfully to direct the protein to the chloroplast however there are no clear rules that will guarantee correct targeting and cleavage of the fusion protein. Some groups have used an exact fusion of the transit peptide to the heterologous protein; for example the transit peptide of the small subunit of rubisco from petunia has been used in this way (Fray et al (2000) Nature Biotech 17, 1017-1020). Others have used transit peptides with two amino acids of the mature protein, eg potato granule-bound starch synthase (GBSS) (Kortsee et al (1996) Plant Journal 10, 83-90), and some have used as many as 23 amino acids of the mature protein eg the small subunit of rubisco from pea (Nawrath et al (1994) Proc. Natl. Acad. Sci USA 91, 12760-12764). Another group used the transit peptide of the Arabidopsis small subunit of rubisco plus 24 amino acids of the mature protein followed by a repeat of six amino acids before the cleavage site and an additional two amino acids of the mature rubisco protein (Wong et al (1992) Plant Mol. Biol. 20, 81-93).

Proteins that enter the secretory system have N-terminal targeting peptides generally known as "signal peptides or sequences" which direct them initially to the endoplasmic reticulum (ER) whence, depending on additional sequences, the proteins are sorted either to vacuoles or are secreted from the cell. In addition, proteins which enter the ER and have a carboxy terminal peptide KDEL are retained within the ER membrane system. For location in the cytoplasm, no targeting sequence is required.

The targeting of classical conventional antibodies (ie IgG, scFv etc) to various cellular compartments of plants has

been reviewed in Conrad and Fielder (1998) Plant Molecular Biology 38, 101-109. Most commonly the homologous signal sequence of the murine IgG (e.g. CEA66E3) has been used to direct expression of the antibody via the secretory pathway, although the signal sequences from plants (PR1a, legumin B4, 2S storage protein) and even bacteria (pelB) and yeast have been used successfully. Generally the highest expression levels were obtained by the use of a signal peptide and C-terminal KDEL ER retention signal. There are no reports of successful targeting of antibodies to chloroplasts using a transit peptide as the targeting sequence. However, Düring et al (1990) Plant Mol. Biol. 15, 281-293, reported that when the barley α-amylase signal peptide was fused to the light and heavy chains of a monoclonal antibody, there was some apparent aberrant localisation of antibody within the chloroplast although the majority of the labelling was in the ER. The authors had no explanation for this observation and were unsure whether import into the chloroplast had actually occurred or if there were other reasons for the specific labelling. Interestingly, no labelling could be detected in the expected location of the apoplastic space or in the golgi apparatus or secretory vesicles.

WO 94/4678 (Casterman et al) describes the isolation of immunoglobulins from Camelids. These immunoglobulins, hereinafter "heavy chain immunoglobulins" have a characteristic distinct structure in that they are naturally devoid of light chains, with the antigen-binding sites being contained entirely within the heavy chain variable domains. This in turn leads to a characteristic structure for the heavy chains of these immunoglobulins, because the variable domain has no interaction sites for a light chain and a complete antigen-binding site has to be formed with no contribution from the hypervariable loops of a light chain variable domain. The heavy chain variable fragments (HCV) of these

immunoglobulins are often referred to as VHH fragments. Such immunoglobulins or fragments thereof, show the functional properties of conventional, four chain, immunoglobulins but by virtue of their simplified structure offer advantages in preparation and use.

There are no reports in the literature of correctly targeted expression in plants of heavy chain immunoglobulins or fragments thereof. A report has appeared in a PhD thesis (Vu, 1999, Vrije Universiteit, Brussels, Belgium) of expression of a heavy chain variable domain in fusion with a chloroplast targeting peptide in *Nicotiana tabacum*. However, targeting to the chloroplast appears to have failed and the subcelluar localisation of the antibody fragments (which remained as a fusion protein) was not established.

There remains a continuing need for the development of improved methods for the production of antibodies and fragments thereof in plants, with the aim of providing methods suitable for economical large scale production for protecting plants from pathogens and for modulating their metabolism.

SUMMARY OF THE INVENTION

Accordingly, in a first aspect the invention provides a method for modifying a plant to produce an antibody or an active fragment or derivative thereof in a desired cellular compartment, comprising introducing into a plant a DNA sequence encoding a heavy chain immunoglobulin or an active fragment or derivative thereof, or a sequence encoding a protein functionally equivalent thereto, said DNA sequence being operably linked to one or more promoters and provided, as appropriate, with an additional sequence encoding a peptide sequence capable of targeting said antibody or fragment or derivative thereof to said desired cellular compartment.

Also provided is a method for preparing a heavy chain immunoglobulin or an active fragment or derivative thereof comprising extracting said immunoglobulin or fragment or derivative thereof from a plant modified according to the first aspect of the invention.

In an alternative aspect, the invention provides the use of a DNA sequence encoding a heavy chain immunoglobulin or an active fragment or derivative thereof, or a sequence encoding a protein functionally equivalent thereto, to cause a plant to produce said antibody or fragment or derivative thereof or protein functionally equivalent thereto in a desired cellular compartment.

Also provided is a modified plant having, in a desired cellular compartment, enhanced levels of heavy chain immunoglobulins or active fragments or derivatives thereof, or proteins functionally equivalent thereto, particularly compared to equivalent but unmodified plants.

Seeds, fruits and progeny of such plants and hybrids are included within the invention.

Food products such as sauces, dressings, tomato products such ketchups, meals, juices and soups, comprising a plant or part thereof according to the invention are also provided.

Also provided are skin and hair protective compositions and pharmaceutical compositions comprising a plant or part thereof according to the invention.

As used herein, "plant" means a whole plant or part thereof, or a plant cell or group of plant cells, or an extract thereof. The invention is particularly directed at

transforming whole plants and the use of the whole plant or significant parts thereof.

The term "antibody" refers to an immunoglobulin which may be derived from natural sources or synthetically produced, in whole or in part. An "antibody fragment", alternatively an "active fragment", is a portion of a complete antibody or immunoglobulin which retains the ability to exhibit at least part, and preferably all, of the antigen binding activity.

A "heavy chain immunoglobulin" is an immunoglobulin naturally devoid of any variable light chain domains but which is capable of specifically combining with an antigen. The antigen-binding capacity and specificity is located exclusively in the immunoglobulin heavy chains, more specifically in the heavy chain variable domains.

The "sequence encoding the heavy chain immunoglobulin or an active fragment or derivative thereof" encompasses a genomic or cDNA clone or a sequence which in proper reading frame encodes an amino acid sequence which is functionally equivalent to the amino acid sequence of the protein encoded by the genomic or cDNA clone. By "functionally equivalent" is meant any protein or fragment or derivative thereof which has the same or similar antigen-binding properties, said antigen-binding capacity being located in a single binding domain. It should be understood, however, that isolated VH domains of conventional antibodies are not included within the scope of the invention. Similarity in functionality can be evaluated by routine screening assays, for example, by assaying the binding affinity of the immunoglobulins produced upon expression in plants.

A "functionally equivalent" derivative may be characterised by an insertion, deletion or substitution of one or more amino

acid residues in the sequence of the heavy chain immunoglobulin or active fragment thereof and includes within its scope fusion molecules. Such derivatives may readily be made by using conventional techniques well known in the art such as peptide synthesis techniques or recombinant DNA manipulation of a gene encoding a heavy chain immunoglobulin by site directed mutagenesis.

It will be appreciated that immunoglobulins or fragments or derivatives thereof modified to enable them to function as binding domains in the same way as immunoglobulins naturally devoid of light chain domains ("heavy chain immunoglobulins") may also suitably be used according to the invention.

A "gene" is a DNA sequence encoding a protein, including modified or synthetic DNA sequences or naturally occurring sequences encoding a protein, and excluding the 5′ sequence which drives the initiation of transcription.

"Operably linked to one or more promoters" means the gene, or DNA sequence, is positioned or connected to the promoter in such a way to ensure its functioning. The promoter is any sequence sufficient to allow the DNA to be transcribed. After the gene and promoter sequences are joined, upon activation of the promoter, the gene will be expressed.

An "equivalent, unmodified" plant is a plant which has a substantially identical genotype to a modified plant of the invention excepting the introduced sequences present in the plant of the invention.

A "construct" is a polynucleotide comprising nucleic acid sequences not normally associated in nature.